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A new 'sense' RNA approach to block viral RNA replication *in vitro*

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ABSTRACT

The use of "antisense" RNA is being widely considered to block specific steps in viral infection. We propose here a new "sense" RNA approach to block viral RNA replication *in vitro* and possibly *in vivo* . In the turnip yellow mosaic virus (TYMV) system, the recognition site of the viral replicase (RNA-dependent RNA polymerase) is assumed to be located within the 3' end of the RNA genome. Small "sense" RNAs have been obtained by *in vitro* transcription of the corresponding cloned cDNAs. Replication of TYMV RNA *in vitro* is shown here to be blocked only by those RNAs that contain the 3' terminal region of the genome.

INTRODUCTION

Recent developments of antiviral research have focused on the use of complementary oligonucleotides [1] or "antisense" RNAs [2] to block specific steps in viral infection. A new "sense" RNA approach is described here to block replication of a viral genome.

Turnip yellow mosaic virus (TYMV) the type member of the tymoviruses contains, as do most plant viruses, a single-stranded "+" RNA genome. This RNA of 2 x 10^6 daltons (d) possesses a tRNA-like structure at its 3' end that efficiently interacts with many tRNA-specific proteins [3-5]. In RNA viruses one of the recognition sites of the replicase (RNA-dependent RNA polymerase) is assumed to reside at the 3' end of the genome. Two cDNA fragments corresponding to the 3' terminal region of TYMV RNA have been subcloned into an *in vitro* transcription vector so as to produce various small RNAs of the same polarity as the viral genome. These "sense" RNAs are referred to as genome-like fragments (glfs). It is shown here that glfs are able to block replication of TYMV RNA *in vitro* only when they contain the 3' end of the viral genome. We believe that this "sense" RNA approach could be of general potiental use in antiviral research.

MATERIALS AND METHODS

Materials

All chemicals were from Merck except that Tris and Lubrol were from Sigma, and acrylamide, bis-acrylamide and urea were from Serva. Nucleoside triphosphates, *Escherichia coli* $tRNA_I^{Val}$ and calf intestinal alkaline phosphatase special for Molecular Biology were from Boehringer Mannheim, and poly(A)$_{>28}$ was from Sigma. Bovine serum albumin special for

Molecular Biology came from B.R.L., RNAsin, RQ$_1^{TM}$ DNAse, the Gemini system and the restriction enzymes Sau3A, Sma1 and EcoR1 were from Promega Biotec, T4-DNA ligase and the restriction enzyme Dde1 were purchased from Biolabs, the restriction enzyme Bgl2 and Klenow enzyme from Amersham and the restriction enzyme Pvu2 from Appligene. [α-^{32}P]UTP (14.8 TBq/mmol) was from Amersham. TYMV-infected Chinese cabbage leaves were kindly provided by S. Astier-Manifacier (INRA, Versailles) and TYMV was purified by the method of Leberman [6]. The viral RNA was extracted [7] under RNase-free conditions and stored at -70°C. The bacterial strain JM 109 was a kind gift of J. Messing [8].

TYMV replicase preparation

TYMV replicase was isolated from TYMV-infected Chinese cabbage leaves according to Mouchès *et al.* [9] up to and through the "PEG" step [10].

TYMV replicase assay

The conditions were those described by Mouchès *et al.* [9]. A solution (25 µl) containing 40 mM Tris-HCl pH 9 (at 25°C), 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 4% (v/v) 95° ethanol, 0.037% (w/v) Lubrol W, 500 µM each of ATP, CTP and GTP and 12.5 µg of TYMV replicase was preincubated for 5 min at 0°C in the absence or in the presence of one of the glfs, of poly(A) or of *E. coli* tRNA$_1^{Val}$ as indicated; [α-^{32}P]UTP (see Tables for amounts) and TYMV RNA (1 µg) were then added and the solution was incubated for 90 min at 30°C. The cold trichloroacetic acid (TCA) precipitable radioactivity was determined on the total incubation mixture. In certain experiments, incubation was for 15 instead of 90 min at 30°C, and the replication products were analyzed as indicated below.

Analysis of the replication products

The replication products were ethanol-precipitated and the dried pellets were dissolved in 12 µl of a loading buffer containing 5.5 M urea, 0.03 M Tris-borate pH 8.3, 0.66 mM EDTA, 0.02% xylene cyanol and 0.02% bromophenol blue. Denaturing gel electrophoresis was performed on 6% polyacrylamide-7 M urea gels in 0.089 M Tris-borate pH 8.3 and 2 mM EDTA. The gels were run for 20 min at 500 V and then for 45 min at 1000 V.

Subcloning of the 3' fragment of TYMV cDNA into the Gemini system

The construction of the initial plasmids R'89 and B26 that contain respectively 1.5 and 0.9 kilobases (kb) of TYMV cDNA will be described elsewhere (Morch *et al.*, in preparation). These two plasmids mainly differ in that the cDNA in R'89 is followed by a poly(dA-dT) tail of about 50 residues whereas in B26 it is directly juxtaposed to the Sma1 cloning site of the vector.

Two subcloning procedures were used (Fig. 1). Plasmid R'89 was first digested with Sma1. The resulting 322 base pair (bp) fragment was purified on a 7% polyacrylamide gel [11] and further digested by Sau3A. The resulting fragments were made blunt end by fill in reaction and ligated into the pGEM1 vector of the Gemini system previously linearized with Sma1 and treated with alkaline phosphatase. After transformation into JM109, recombinant plasmids were

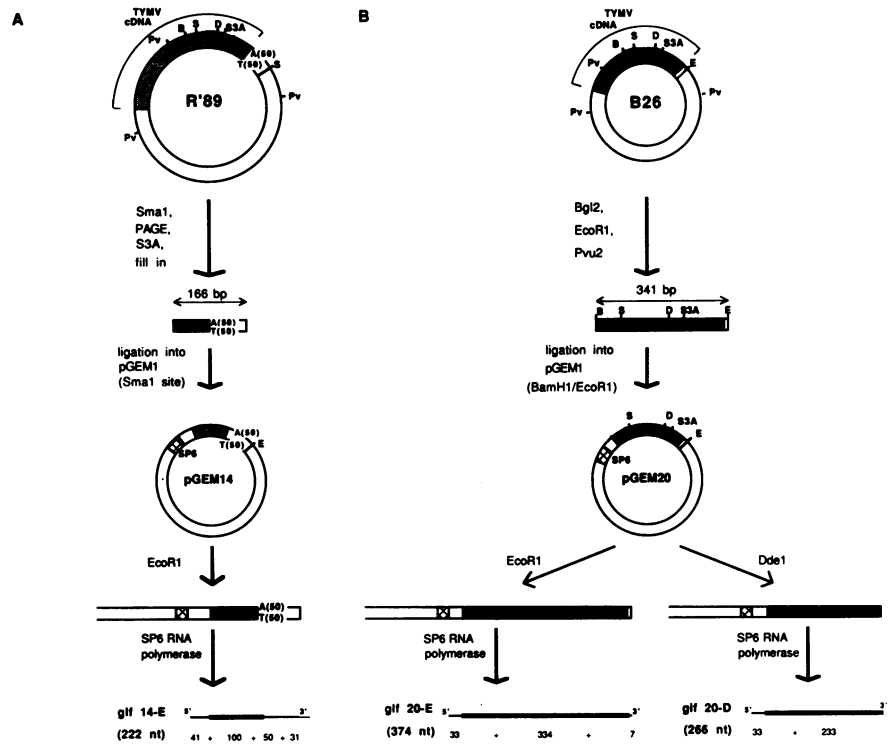

Fig. 1. Strategy adopted to produce SP6 transcripts (glfs) containing the 3' terminal region of TYMV RNA starting from A: plasmid R'89 and B: plasmid B26. ▬ = TYMV cDNA, the dark part corresponding to the 3' terminal fragment to be cloned; ▨ = SP6 promotor of the pGEM1 vector (Gemini system). The length in base pairs (bp) of the DNA fragments cloned is indicated. S = Sma1, S3A = Sau3A, E = EcoR1, D = Dde1, Pv = Pvu2, B = Bgl2 restriction sites. PAGE = polyacrylamide gel electrophoresis. The size in nucleotides (nt) of the RNA produced upon SP6 transcription of the linearized plasmids is indicated below the corresponding stretch. The thicker line in the schematic representation of the transcribed glfs corresponds to the viral sequence.

screened on the basis of a Taq1 digestion assay. The Taq1 restriction pattern (not shown) allowed us to establish which of the two Sau3A fragments had been inserted and to determine their orientation. Construct pGEM14 was chosen and large scale DNA preparation performed according to Holmes and Quigley [12].Plasmid B26 was digested consecutively with Bgl2, EcoR1 and Pvu2. The Bgl2-EcoR1 cDNA fragment is 341bp long. Pvu2 digestion prevented religation into the initial vector and permitted direct ligation of the fragments into pGEM1 linearized with BamH1 and Eco R1. After transformation into JM109, recombinant plasmids were screened on the basis of their size and checked by restriction digestion analysis. Construct pGEM20 was selected and large scale DNA preparation was performed [12].

In vitro transcription

The transcription reactions were performed as recommended by Promega Biotec. Linearized DNA template (2µg) was incubated at 37°C for 90 min in 100 µl containing 50 mM Tris-HCl pH 7.5, 10 mM NaCl, 6 mM $MgCl_2$,10 mM DTT, 2 mM spermidine, 500 µM each NTP, 120 U of RNAsin and 20 U of SP6 RNA polymerase. RQ$_1^{TM}$ DNase (2 U) was added and incubation pursued for 15 min. The RNA transcript was purified by phenol-chloroform and chloroform extraction followed by two ethanol precipitations. It was resuspended in 10 µl of sterile water and stored at -70°C. The quality (one major band) and quantity (≈200 ng/µl) of the transcript were estimated by analysis of an aliquot on a denaturing 6% polyacrylamide-7 M urea gel followed by silver staining [13].

RESULTS AND DISCUSSION

Production of various glfs

The successive steps involved in the production of the different 3' glfs are schematized in Figure 1. Two restriction fragments obtained from two different plasmids were subcloned into the pGEM1 vector of the Gemini *in vitro* transcription system in order to compare the efficiency of various constructions in inhibiting TYMV RNA replication *in vitro* .

The initial plasmids contained TYMV cDNA covering 1.5 kb (R'89) or 1 kb (B26) from the 3' end of the genome cloned into the pUC9 vector for the purpose of sequencing. This cDNA has been fully sequenced on both strands (Morch *et al.*, in preparation). The sequence confirms the presence in both plasmids of the region corresponding to the 3' terminal part of TYMV RNA (including the complete tRNA-like structure of 86 nucleotides [nt]). This region is followed in plasmid R'89 by 50 dA:dT pairs resulting from the cloning procedure [14]. In the case of this plasmid (Fig. 1A), a 166 bp-long DNA fragment containing the 3' terminal 100 nt of the genome was excised by Sma1 and Sau3A digestion and ligated into the linearized pGEM1 vector yielding clone pGEM14. When linearized with EcoR1, this plasmid can be transcribed by the SP6 RNA polymerase into glf 14-E, a small RNA molecule of 222 nt encompassing 41 nt of the vector-derived 5'-flanking sequence, 100 nt of the 3'-terminal sequence of the TYMV genome and 50 A residues followed by 31 nt of the vector-derived 3'-flanking sequence. In the case of plasmid B26 (Fig. 1B), a 341 bp-long Bgl2-EcoR1 restriction fragment was obtained and subcloned into pGEM1 leading to construct pGEM20. This plasmid was linearized either with EcoR1 or with Dde1. In the former case, transcription with the SP6 RNA polymerase yielded glf 20-E, a 374 nt-long RNA fragment encompassing 33 nt of the vector-derived 5'-flanking sequence, 334 nt of the 3' terminal sequence of the TYMV genome and 7 nt of the vector-derived 3'-flanking sequence. In the latter case, the SP6 transcript obtained was glf 20-D which exactly corresponds to glf 20-E except for the deletion of the last 108 nt.

Blocking effect of glfs on TYMV RNA replication *in vitro*

Table 1 presents the results obtained in replication assays performed in the presence of

Table 1. Effect of the addition of different glfs on TYMV RNA replication *in vitro*

ADDITIONS (in ∿10x molar excess over TYMV RNA)	REPLICATION (cpm/incubation)*
None	91540
glf 14-E	6822
glf 20-E	6840
glf 20-D	89340

* ∿360 KBq of [α-^{32}P]UTP were used per incubation; blank values obtained in the absence of added TYMV RNA have been substracted for all values.

various glfs in ∿10-fold molar excess over TYMV RNA. The addition of glf 14-E or glf 20-E leads to an important decrease in the efficiency of TYMV RNA replication *in vitro* . On the contrary, TYMV RNA replication is only slightly affected by the presence of glf 20-D. These data show that the glfs containing the 3' terminal nt of the viral genome can efficiently block TYMV RNA replication *in vitro* . This effect depends on the presence in the transcript of a region delimited here by the last 100 nt of the genome; these nt are absent from glf 20-D. Moreover these results indicate that the 100 nt-long region required for inhibition of replication does not need to be located at the 3' end of the transcript: glf 14-E and glf 20-E have the same efficiency of inhibition even though in both transcripts the 100 genomic nt are followed by additional residues: 81 and 7 nt respectively.

To investigate the specificity of inhibition by glf 14-E, a second set of measurements was performed. The results presented in Table 2 indicate that neither poly(A) nor *E. coli* $tRNA_1^{Val}$ significantly affect the efficiency of TYMV RNA replication *in vitro* . This shows that the inhibitory effect of glf 14-E is not due to the poly(A) stretch it contains and that this effect is specific of the viral RNA sequence since it cannot be mimicked by $tRNA_1^{Val}$. This tRNA was chosen because glf 14-E and glf 20-E contain the sequence corresponding to the tRNA-like structure of TYMV RNA, and because the question remained as to whether this structure is required for

Table 2. Comparison of the inhibition efficiencies of various RNAs on TYMV RNA replication *in vitro*

ADDITIONS (in ∿10x molar excess over TYMV RNA)	REPLICATION (cpm/incubation)*
None	43225
glf 14-E	2280
Poly(A)	45740
E. coli $tRNA_1^{Val}$	39695

* ∿90 KBq of [α-32P]UTP were used per incubation; blank values obtained in the absence of added TYMV RNA have been substracted from all values.

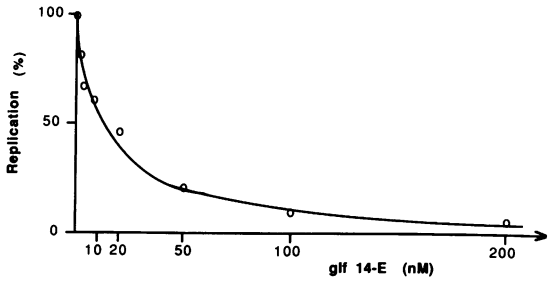

Fig. 2. Effect of increasing amounts of glf 14-E on TYMV RNA replication *in vitro* . Replication is expressed as the percentage of the value obtained in the absence of glf 14-E. 100% = 41400 cpm of [α-^{32}P]UMP incorporated.

replication. Indeed, results obtained with mutagenized brome mosaic virus (BMV) RNAs indicate that the requirements for aminoacylation and replication can be dissociated *in vitro* [15]. Our results also suggest that interaction with the replicase does not rely on the tRNA-like properties of the viral genome. Finally, SP6 transcripts deriving from totally unrelated sequences such as phage λ sequences were also shown to be without effect on the replication of TYMV RNA (not shown).

Effect of glf 14-E concentration on TYMV RNA replication *in vitro*

Increasing amounts of glf 14-E were added to a replication assay performed with a constant TYMV RNA concentration ($\sim$20 nM) and the efficiency of replication determined. Figure 2 shows the percentage of inhibition observed at various glf 14-E concentrations. Inhibition of 50% is obtained at a glf 14-E concentration between 10 and 20 nM, thus in the same concentration range as TYMV RNA. This value suggests that the inhibition observed could result from competition between glf 14-E and TYMV RNA for binding to the replicase, both RNAs having a similar affinity for this enzyme. Replicase bound to 3'-glf would thus be diverted from replicating TYMV RNA and the efficiency of replication would be decreased. However, from these results the question still remained as to whether the replicase bound to the glf could use it as template.

Analysis of TYMV RNA replication products in the presence of glfs

To further investigate the mechanism involved in the inhibition of TYMV RNA replication by glfs *in vitro* , the distribution of the replication products was analyzed on denaturing gels. Figure 3 presents the results obtained using glf 14-E, glf 20-E and glf 20-D on panels A, B and C respectively. It is clear that addition of glf 14-E or glf 20-E leads to a marked decrease in the synthesis of TYMV-specific RNAs whereas in the presence of glf 20-D, this synthesis is virtually unaffected. Moreover, inhibition of TYMV RNA replication is accompanied in the first two cases by the synthesis of one or more additional replication products that correspond to the products obtained when these glfs are incubated alone with the replicase (panels A and B, lanes 6). Glf 20-D which does not inhibit replication, does not give rise to such replication products

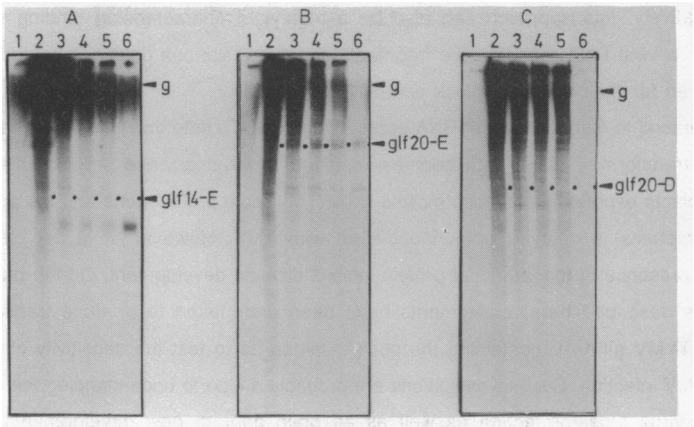

Fig. 3. Analysis of ^{32}P-labeled replication products on 6% polyacrylamide - 7 M urea gels. Panels A, B and C are autoradiograms of three parallel sets of experiments performed using glf 14-E, glf 20-E and glf 20-D respectively. Aliquots (equal volumes) of the replication assays are analyzed after 15 min of incubation under the conditions described in Materials and Methods. For each panel, the templates for the replicase are no RNA (lane 1), TYMV RNA (≈20 nM) without (lane 2) or with (lanes 3 to 5) increasing amounts (see below) of glf and glf alone (lane 6). The concentrations of glf are in panel A (glf 14-E): lane 3, 50 nM; lane 4 and 6, 250 nM; lane 5, 500 nM; in panel B (glf 20-E): lane 3, 30 nM; lanes 4 and 6, 150 nM; lane 5, 300 nM; in panel C (glf 20-D): lane 3, 40 nM; lanes 4 and 6, 200 nM; lane 5, 400 nM. The position of the glf and of TYMV genomic RNA (g) is indicated to the right of each panel.

(panel C, lane 6). Thus it appears that both glf 14-E and glf 20-E can serve as template for the TYMV replicase whereas glf 20-D cannot. These results suggest that the 3' terminal 100 nt of TYMV RNA are not only required for interaction with the replicase but constitute a signal of sufficient length to initiate replication. With glf 14-E, the major replication product migrates as an RNA shorter than glf 14-E itself, suggesting that replication could have been initiated at an internal site, possibly upstream of the poly(A) stretch.

In conclusion, the data presented here indicate that inhibition of viral RNA replication can be obtained by a small RNA fragment of the same polarity as the viral genome, thus by a "sense" RNA.

Concluding remarks

The "sense" RNA approach described here can probably be extended to several other RNA viruses. The only prerequisite is to know where the recognition site of the replicase resides. The most likely site for the initiation of '-' strand synthesis is in the 3' part of the viral genome. This assumption is directly supported by data on BMV RNA replication where mutations introduced into the 3' region of the viral RNA can inhibit initiation of replication *in vitro* [15]. Indirect evidence also comes from *in vivo* studies where alterations in the 3' end affect accumulation of BMV RNA [16], and from the observation of sequence homologies in the 3' end of the RNAs of viruses with a multipartite genome [17-23] and between certain viral RNAs and their satellites

[24]. Alternatively, this approach can also be a means of characterizing binding sites for the replicase on a viral RNA: genome-like fragments containing various deletions could be produced and compared for their ability to block viral RNA replication.

We intend to test our "sense" RNA approach *in vivo* . To date only one report has stated that genetically transformed plants can become resistant to virus disease [25]. In this report, the transgenic plants express the tobacco mosaic virus (TMV) coat protein gene and the appearance of disease symptoms is delayed upon inoculation with TMV. However, it is not clear how the constitutive presence of the viral coat protein inhibits disease development. On the basis of the *in vitro* results described here, experiments have been undertaken to produce transgenic plants expressing TYMV glf 14-E containing the poly(A) tail so as to test the sensitivity of these plants towards TYMV infection. Such investigations are probably a clue to understanding the mechanisms involved in virus cross-protection as well as an open door to new developments in antiviral research.

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